Biogenic amine production by Gram-positive bacteria isolated from Spanish dry-cured "chorizo" sausage treated with high pressure and kept in chilled storage

B. de las Rivas [a], C. Ruiz-Capillas [b], A.V. Carrascosa [a], J.A. Curiel [a], F. Jiménez-Colmenero [b], R. Muñoz [a,*]

[a] *Departamento de Microbiología, Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid*

[b] *Departamento de Ciencia y Tecnología de Carnes y Pescados, Instituto del Frío, CSIC, José Antonio Novais 10, 28040 Madrid*

*Corresponding author. Tel.: +34-91-5622900; fax: +34-91-5644853

E-mail address: rmunoz@ifi.csic.es (R. Muñoz)

Abstract

We studied the production of biogenic amines by 200 strains of lactic acid bacteria and staphylococci isolated during chilled storage from samples of Spanish dry-cured "chorizo" sausage treated with high-pressure. The presence of biogenic amines in a decarboxylase synthetic broth was confirmed by ion-exchange chromatography. β-phenylethylamine was the biogenic amine more frequently produced (22.5%), followed by tyramine (7.5%). In tyramine producer-strains the presence of a tyrosine decarboxylase gene was confirmed by PCR. Among lactic acid bacteria, the production of tyramine was mainly related to the species *Lactobacillus curvatus*. Most of the *L. curvatus* strains were also β-phenylethylamine-producers. In relation to staphylococci, tyramine-production was mainly associated to *Staphylococcus carnosus* strains. The *S. carnosus* strains analysed in this study produced β-phenylethylamine or β-phenylethylamine and tyramine simultaneously. RAPD-PCR results indicated that the biogenic amine-producer *S. carnosus* population changes along storage independently of the high-pressure treatment.

Keyworks: Gram-positive bacteria; Spanish dry-cured "chorizo" sausage; Lactic acid bacteria; Coagulase-negative staphylococci; Biogenic amines; Tyramine; High pressure; Chilled storage

1. Introduction

"Chorizo" is a typical dry fermented sausage from Spain. The basic ingredients of chorizo are lean pork, pork fat, salt and spices (garlic, oregano and red pepper). It is stuffed into natural or artificial casings and ripened at low temperature (24-12 ºC) and relative humidity (from 96 to 65%). Lactic acid bacteria (LAB) and staphylococci are the two main groups of bacteria that are considered technologically important in the fermentation and ripening of "chorizo". LAB are responsible for lactic acid production, and for the small amounts of acetic acid, ethanol, acetoin, carbon dioxide, and pyruvic acid that are produced during fermentation, depending on the carbohydrate used, and the sources of meat proteins and additives. Staphylococci are important for colour stabilization, decomposition of peroxides and aroma with their proteolytic and lipolytic activities (Silla, 1989).

The safety of "chorizo" for consumers could depend partially on the content of biogenic amines (BA), such as histamine, tyramine, putrescine, and cadaverine, which might represent a food poisoning hazard. BA are organic molecules with low molecular weight, biologically active and normally formed by bacterial decarboxylation of their precursor amino acids. These molecules are generally either psychoactive or vasoactive, and furthermore, putrescine and cadaverine have been described as precursors of carcinogenic nitrosamines (Wathersen, Scanlan, Bills, & Libbey, 1975). Dry cured sausages can potentially support the accumulation of BA (Ruíz-Capillas & Jiménez-Colmenero, 2004). In fact, the high amounts of proteins present in these products and the proteolytic activity during ripening provide the precursors for decarboxylase activity of microbiota. The production of BA requires the presence of amino-acid decarboxylating microorganisms possessing a different ability in synthesizing

69 decarboxylases (Silla, 1996). Within the same species, the presence, the activity, and the
70 specificity of decarboxylases is strain-specific (Suzzi & Gardini, 2003).
71 Futhermore, BA formation may be influenced by the technological conditions used
72 to produce and store the "chorizo" sausage. So, alternative non-thermal technologies
73 show challenging possibilities in this connection. For instance, high pressure processing
74 is a preservation method that kills and/or sub-lethally injures microorganisms mainly
75 due to the membrane damage. Previously, we have studied BA formation and
76 microbiota evolution in Spanish dry-cured "chorizo" sausage treated with high-pressure
77 and kept at 2 ºC (Ruíz-Capillas, Jiménez-Colmenero, Carrascosa, & Muñoz, 2007).
78 Since i) the production of BA in dry-cured sausages has been attributed to the action of
79 different groups of microorganisms such as *Enterobacteriaceae*, LAB and
80 staphylococci, ii) the *Enterobacteriaceae* levels were low, indicating a good hygienic
81 manufacturing conditions (Ruíz-Capillas et al., 2007), we decided to examine the
82 occurrence of amino-acid decarboxylase activity in Gram positive strains (LAB and
83 staphylococci) isolated from the samples obtained during the storage of "chorizo"
84 treated with high-pressure.
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87 **2. Materials and methods**
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89 *2.1. Sampling procedure, strain isolation and growth conditions*
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91 Spanish dry-cured "chorizo" sausage samples treated with high-pressure and kept in
92 chilled storage were prepared as described previously (Ruíz-Capillas et al., 2007).
93 Briefly, slices (3 mm thick) were taken from commercial "chorizo" under strict hygienic

94 conditions. Slices packed in plastic bags (WIPAK® PAE 110KFP) were divided in two
95 batches. One of the batches (CHP) was pressurized at 350 MPa/15 min, in an ACB
96 model AGIP No. 665 high-pressure pilot unit (GEC, Alsthom, Nantes, France) using
97 water at 20 ºC as the pressuring medium. The other batch was not pressurized (CH) and
98 used as control. Both batches were kept at 2 ± 1 ºC. Samples were withdrawn at 0, 34,
99 74 and 160 days of chilled storage, and homogenized in a stomacher blender
100 (Stomacher Colworth 400, Seward, UK). The homogenised samples were plated on
101 MRS agar (Difco, France) and on Mannitol Salt Agar (MSA) (Difco, France) for LAB
102 and *Micrococcaceae* colony counting.
103 A total of two hundred gram-positive bacteria colonies (LAB and staphylococci)
104 were assayed for BA production. Twenty-five representative strains were selected from
105 each "chorizo" sample (CHP or CH) in every storage time. From the 25 colonies of each
106 sample, 20 colonies were picked from high dilution MRS plates and five colonies from
107 MSA plates. Production of BA was tested by inoculating individual colonies from MRS
108 or MSA plates directly into tubes containing the modified decarboxylase medium
109 described by Maijala (1993). Pyridoxal-5-phosphate was included in the medium (at
110 0.005%) since its a cofactor for the decarboxylation reactions. The medium contained
111 the corresponding precursor amino acids (L-histidine monohydrochloride, L-ornithine
112 monohydrochloride, L-lysine and L-arginine monohydrocloride at 0.25% final
113 concentration, and tyrosine disodium salt at 0.2% due to its low solubility). Purple
114 bromocresol was included as pH indicator. The pH was adjusted to 5.3 and the medium
115 was autoclaved. The precursor amino acids were purchased from Sigma (St. Louis, MO,
116 USA). The inoculated tubes were incubated at 30 ºC during 7 days.

For DNA extraction, LAB and staphylococci strains were routinely grown in MRS media and in brain heart infusion (BHI, Difco, France), respectively, at 30 ºC under microaerobic conditions.

2.2. Biogenic amine analysis from bacterial cultures by ion-exchange chromatography

Bacterial strains were grown in the modified Maijala´s medium as described in the 2.1 section. After incubation, 1 ml of the broth media was centrifuged (12,000 x *g*, 5 min), then 0.5 ml of supernatant was extracted with 0.5 ml of 0.1 N HCl, centrifuged again (12,000 x *g*, 5 min), and filtered through 0.22 nm. The extract was analysed by ion exchange chromatography for BA content. Tyramine, phenylethylamine, histamine, putrescine, cadaverine, agmatine and spermidine were determined following the methodology of Ruíz-Capillas and Moral (2001) in a HPLC model 1022 with a Pickering PCX 3100 post-column system (Pickering Laboratories, Mountain View, CA, USA).

2.3. Presence of a tyrosine decarboxylase gene in the tyramine-producer strains

Bacterial chromosomal DNA was isolated from overnight cultures using a protocol previously described (Vaquero, Marcobal, & Muñoz, 2004). Chromosomal DNAs from the tyramine-producer strains were subjected to PCR amplification to detect the presence of the tyrosine decarboxylase encoding gene (*tdc*) (Landete, de las Rivas, Marcobal, & Muñoz, 2007). We used two oligonucleotide sets previously described to

amplify the *tdc* gene in the tyramine-producer strains. Firstly, we used primers P1-rev (5´-CCRTARTCNGGNATAGCRAARTCNGTRTG) and P2-for (5´-GAYATNATNGGNATNGGNYTNGAYCARG) that amplified a 924-bp DNA fragment from the *tdc* gene in LAB. In addition, primer set TDC-F (5´-TGGYTNGTNCCNCARACNAARCAYTA) and TDC-R (5´-ACRTARTCNACCATRTTRAARTCNGG) that amplified an 825-pb *tdc* DNA fragment was also used (being Y = C or T; R = A or C, and N = A, C, G, or T) (Landete et al., 2007). PCR reactions were performed in 0.2 ml microcentrifuge tubes in a total volume of 25 µl containing 1µl of template DNA (aprox. 10 ng), 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1µM of each primer, and 1 U of Ampli*Taq* Gold DNA polymerase. The reaction was performed in a GeneAmp PCR System 2400 (Perkin Elmer, USA) using the following cycling parameters: 10 min for enzyme activation at 95 ºC followed by 30 cycles of 30 s at 95 ºC, 30 s at 53 ºC, and 2 min at 72 ºC, and a final extension step of 20 min at 72 ºC. PCR products were resolved on a 2% agarose gel (Pronadisa, Spain) and stained with ethidium bromide.

2.4. Taxonomical identification of the biogenic amine-producer strains.

BA-producer strains were identified by PCR amplification and DNA sequencing of their 16S rDNA. The16S rDNAs were PCR amplified using the eubacterial universal pair of primers 63f (5´-CAGGCCTAACACATGCAAGTC) and 1387r (5´-GGGCGGWGTGTACAAGGC) previously described (Marchesi et al., 1998). The 63f and 1387r primer combination generates an amplified product of 1.3 kb. PCR was performed in 25 µl amplification reaction mixture as described above. The reaction was

167 performed by using the following cycling parameters: initial 10 min for enzyme
168 activation at 95 ºC followed by 35 cycles of 1 min at 94 ºC, 1 min at 50 ºC and 1:30 min
169 at 72 ºC. Amplified products were resolved on a 0.7% agarose gel. The amplifications
170 products were purified on QIAquick spin Columns (Quiagen, Germany) for direct
171 sequencing. DNA sequencing was carried out by using an Abi Prism 377^{TM} DNA
172 sequencer (Applied Biosystems, USA). Sequence similarity searches were carried out
173 by comparing to sequences from type strains included on the Ribosomal Database.
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176 *2.5. Intraspecific discrimination of biogenic amine-producer strains*
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178 The BA-producer strains belonging to the *Staphylococcus carnosus* species were
179 discriminated by a random amplified polymorphic DNA (RAPD) typing method.
180 Approximately 10 ng of chromosomal DNA was subjected to PCR amplification in the
181 reaction mixture described above, but containing only one oligonucleotide in the
182 mixture. The reaction mixtures were subjected to amplification at the following
183 conditions: initial 10 min for enzyme activation at 95 ºC followed by 40 cycles of 1 min
184 at 94 ºC, 1 min at 36 ºC and 2 min at 72 ºC. The oligonucleotide selected for RAPD
185 analysis was the M13 minisatellite core sequence (5´-GAGGGTGGCGGTTCT) (Huey
186 & Hall, 1989).
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189 **2. Results and discussion**
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High-pressure treatment prolongs the shelf life of products under strict temperature; therefore, in a product treated in this way, the evolution of microbiota may affect the presence of BA. Generally, *Enterobacteriaceae* are rarely found in ripening meat products due to their high sensitivity to acidity and desiccation (Lücke, 1986). In addition, we have demonstrated that in the Spanish dry-cured "chorizo" sausage treated with high pressure and kept in chilled storage, *Enterobacteriaceae* level remains below 2 log cfu/g (Ruíz-Capillas et al., 2007). Therefore, we only studied the ability to produce BA by the main microorganism groups present in these samples.

Several qualitative and quantitative methods to determine the production of BA by microorganisms have been described. Most of the screening procedures generally involve the use of a differential medium containing a pH indicator. The pH change is dependent on the production of the more alkaline amine form the amino acids initially included in the medium. However, false-positive and false-negative results have been described in these media (Marcobal, de las Rivas, & Muñoz, 2006a). In order to facilitate the growth of meat LAB, Maijala (1993) developed a modified decarboxylase media. A total of two-hundred Gram-positive bacteria, including LAB and staphylococci, were tested for BA-production in the Maijala´s decarboxylase medium (1993) and all were able to growth in this medium. BA-positive reactions were recorder when a purple color was formed in the decarboxylase broth. We evaluated the suitability of the medium by confirmation of the quantitative amine-forming capability using a chromatographic assay. As showed in Table 1, correlation was not found between purple positive tubes and the presence of BA. In most cases, the decarboxylase medium underestimates the number of BA-producer strains, giving false-negative results that could be produce by an insufficient growth of the strains. On the other hand, false-positive results were obtained in two samples, on which the number of BA-positive

strains was lower than the positive tubes containing decarboxylase medium. This could be due to the production of a substance able to alkalinize the media since when these cultures were analyzed for the presence of BA by HPLC none of them showed BA production. The results obtained in this work confirmed previous results describing that false-positive and false-negative results could be obtained from the decarboxylase medium.

From Table 1 it could be deduced than only 31 out 200 strains (15.5%) were presumptively detected as BA-produced in decarboxylase medium. However, 45 out 200 strains analyzed (22.5%) were confirmed to be able to produce BA. A lower incidence of BA-producer strains was previously reported for lactobacilli and staphylococci isolated from slightly fermented sausages, since among 250 LAB strains, only 39 strains (15.6%) produced one or more BA (Aymerich, Martín, Garriga, Vidal-Carou, Bover-Cid, & Hugas, 2006), and among 240 staphylococci, a total of 35 strains (14.6%) were able to decarboxylate one or more amino acids (Martín, Garriga, Hugas, Bover-Cid, Veciana-Nogués, & Aymerich, 2006).

β-phenylethylamine was the BA more frequently produced (22.5%), followed by tyramine (7.5%). Putrescine and cadaverine were only produced by the 1.5 and 0.5%, respectively, of the strains analyzed (Table 1). It is remarkable that β-phenylethylamine was produced by all the BA-producer strains. These strains produce only β-phenylethylamine or β-phenylethylamine together with other BAs. As expected, histamine-producer bacteria were not detected, since bacteria with the capacity to decarboxylate histidine are uncommon in meat (Paulsen & Bauer, 1997). The proportion of β-phenylethylamine- and tyramine-producer strains is higher than those reported previously. Previous works on slightly fermented sausages found that, among LAB, tyramine was produced by 14.4% of the strains, followed by β-phenylethylamine

241 (12.4%), and putrescine (0.8%) (Aymerich et al., 2006), and among staphylococci, β-
242 phenylethylamine was the BA more frequent detected (10.8%), followed by tyramine
243 (4.6%), cadaverine (2.9%), histamine (1.3%) and tryptamine (0.4%) (Martín et al.,
244 2006).

245 In order to correlate the production of tyramine with the presence of the
246 corresponding tyrosine decarboxylase gene, we performed PCR assays for the detection
247 of the *tdc* gene. Since several molecular methods have been described to detect
248 tyramine-producer bacteria, we checked the presence of the *tdc* gene by using two
249 oligonucleotide sets, primer P1-rev + P2-for and TDC-F + TDC-R (Landete et al.,
250 2007). As showed in Figure 1, tyramine-producer strains gave the corresponding
251 amplicon of the expected size, so, a tyrosine decarboxylase gene was present on them.
252 Tyrosine decarboxylase genes have been detected in tyramine-producer LAB such as
253 *Lactobacillus brevis*, *Enterococcus faecium*, *Carnobacterium divergens*, among others
254 (Landete et al., 2007). Recently, a partial *tdc* gene sequencing from a *L. curvatus* strain
255 isolated from slightly fermented sausages was carried out (Aymerich et al., 2006).

256 Since the production of BA was confirmed by chromatographic and molecular
257 methods, we decided to taxonomically identify the bacteria producing tyramine in this
258 study. Phenotypic identification of fermentative microbiota is time-consuming and often
259 problematic due to ambiguous biochemical or physiological traits. The development of
260 molecular methods has offered the possibility of accelerating a great deal of bacterial
261 identification. Therefore, the taxonomical identity of the tyramine-producer strains was
262 assessed by the amplification of the DNA fragment coding the 16S rDNA. The bacterial
263 isolated identified as positive for BA-production were then identified using sequence
264 data from the first 500 bp of the 16S rRNA genes. The sequences obtained were
265 compared to sequences from type strains included on the Ribosomal Database. The

266 tyramine-producer strains were identified belonging mainly to the *Lactobacillus*
267 *curvatus*, and *Staphylococcus carnosus* species, although some *Lactobacillus*
268 *plantarum*, *Lactobacillus brevis*, and *Staphylococcus warneri* strains were also
269 tyramine-producers (Fig. 1).

270 Among meat LAB, the production of tyramine was mainly related to the species *L.*
271 *curvatus* (Straub, Kicherer, Schilcher, & Hammes, 1995). In addition, among LAB
272 isolated from slightly fermented sausages, most of the tyramine-producer strains were
273 identified as member of this species (Aymerich et al., 2006). The tyramine-producer *L.*
274 *curvatus* strains isolated in this work were also β-phenylethylamine-producers, as also
275 reported by Aymerich et al. (2006). We described previously that the tyrosine-
276 decarboxylase enzyme from *E. faecium* is able to decarboxylate tyrosine as well as L-
277 phenylalanine, producing tyramine and β-phenylethylamine, respectively (Marcobal, de
278 las Rivas, & Muñoz, 2006b). So, it could be assumed that a similar decarboxylase might
279 be found in *L. curvatus*.

280 In relation to staphylococci, tyramine-production was mainly associated to
281 *Staphyloccus carnosus* strains (Fig.1). It is noteworthy, that most of the only β-
282 phenylethylamine-producers strains were also *S. carnosus.* The *S. carnosus* strains
283 isolated in this work produced β-phenylethylamine or β-phenylethylamine and tyramine
284 simultaneously. Previously, all *S. carnosus* strains isolated from slightly fermented
285 sausages were reported to produce β-phenylethylamine, without producing tyramine or
286 other amines (Martín et al., 2006). However, *S. carnosus* strains isolated from Slovak
287 meat products, produced primarily tyramine, but also β-phenylethylamine (Simonová et
288 al., 2006). We have amplified a DNA fragment of the *tdc* gene encoding a putative
289 tyrosine decarboxylase in *S. carnosus* strains (Fig. 1), however, in *S. carnosus*, the
290 enzyme responsible for the biosynthesis of both amines remains unknown. Therefore,

291 we could only speculate about a different substrate affinity for both amino acids,
292 tyrosine and phenylalanine, between *L. curvatus* and the *S. carnosus* decarboxylase,
293 e.g., showing the *S. carnosus* decarboxylase highest affinity to phenylalanine and the *L.*
294 *curvatus* enzyme to tyrosine. No differences were observed between bacterial species
295 producing BA in pressurized samples as compared to the non-pressurized ones.
296 Since most of the BA-producer strains found in this work were identified as *S.*
297 *carnosus,*, we decided to know the intraspecific diversity existing on these BA-strains.
298 Among PCR- based techniques, it is widely recognised that RAPD-PCR could be a
299 rapid and reliable method for typing strains. Genomic DNA from representative *S.*
300 *carnosus* strains was used as template for PCR fingerprinting using as a primer the M13
301 minisatellite core sequence (Huey & Hall, 1989). The M13 sequence has successfully
302 been used to obtain strain-specific patterns of many bacterial meat species (Rossetti &
303 Giraffa, 2005; Rantsiou, Drosinos, Gialitaki, Metaxopoulos, Comi, & Cocolin 2006).
304 Fig. 2 showed the genetic heterogeneity found among *S. carnosus* strains as observed by
305 the diversity of RAPD-M13 patterns obtained. Genetic diversity among *S. carnosus*
306 strains was also reported previously (Aznar & Chenoll, 2006). It is also remarkable that
307 the *S. carnosus* population seems to change along storage, since strains isolated at the
308 treatment time shared the same RAPD-M13 pattern (Fig. 2). The evolution of *S.*
309 *carnosus* population seems to be not associated with the high pressure treatment as
310 pattern evolution along storage was observed in pressurized as well as in non-
311 pressurized samples.
312 Many procedures have been proposed to evaluate the decarboxylase activity of
313 bacteria isolated from foods. Within the same species, the presence, the activity and the
314 specificity of decarboxylases is strain-specific. Rapid screening methods can have some
315 limitation in terms of sensitivity in detecting BA production leading to contradictory

316 results. For these reason, BA production has to be confirmed by analytical quantitative
317 methods. Moreover, the negative (or positive) responses in screening media do not
318 necessarily imply a similar behaviour in food products. In spite that *L. sakei* and *S.*
319 *xylosus* species, among LAB and staphylococci, are the most frequently species isolated
320 from chorizo samples, they mainly possess non BA-producer strains as revealed in this
321 study. Producers of dry fermented meat products should test starter cultures for
322 decarboxylase activity.
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Figure captions

Fig. 1. Amplification by PCR of the tyrosine decarboxylase gene (*tdc*) in bacteria isolated from dry-cured "chorizo" sausage treated with high-pressure. Primer set P1-rev and P2-for that amplified a 924-bp *tdc* fragment was used on lanes a to d; similarly, lanes 5 to 9 showed the amplification of a 825-bp *tdc* fragment by using TDC-R and TDC-F oligonucleotide primers. DNA from some tyramine-producer strains belonging to the *Staphylococcus carnosus* (lane a), *Staphylococcus warneri* (lane b), *Lactobacillus plantarum* (lanes c to e), *Lactobacillus curvatus* (lanes f to h), and *Lactobacillus brevis* (lane i) species was used in the PCR. The molecular sizes of some standards (*Eco*RI/*Hin*dIII-digested λ DNA) are indicated on the left.

Fig. 2. RAPD patterns obtained with primer M13 on *Staphylococcus carnosus* strains isolated from dry-cured "chorizo" sausage treated with high-pressure and kept in chilled storage. Strains were isolated from samples withdrawn at day 0 (lanes a to e), 34 days (lanes f, and g), 74 days (lanes h, and i), and 160 days (lanes j to m) of chilled storage. A DNA marker standard (EcoRI/HindIII digested λ DNA) was included on the right.

Table 1

Table 1
Biogenic amine production by Gram-positive bacteria isolated from "chorizo" treated with high-pressure and kept in chilled storage.

		Strains producing biogenic amines				
			HPLC[c]			
Days[a]	Sample	MD[b]	PEA	TYR	PUT	CAD
0	CH	3	9	6	0	0
	CHP	4	7	3	0	0
34	CH	4	5	0	0	0
	CHP	14	11	1	0	0
74	CH	2	7	5	3	1
	CHP	3	2	0	0	0
160	CH	1	1	0	0	0
	CHP	0	3	0	0	0

[a] Days of storage at 2 ± 1 ºC.
[b] MD, number of positive strains in modified decarboxylase broth
[c] HPLC, number of positive strains by ion-exchange chromatography
PEA, β-phenylethylamine; TYR, tyramine; PUT, putrescine; CAD, cadaverine.

Figure 1

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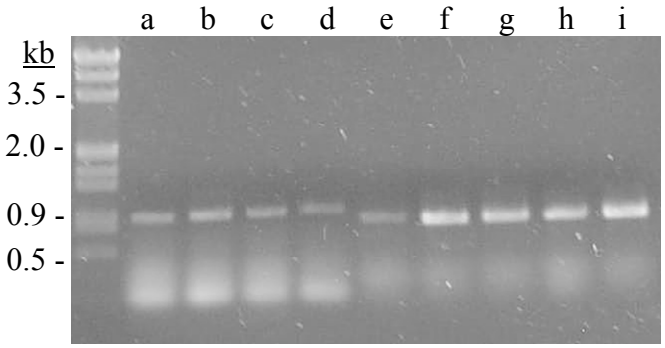

Figure 2

Figure 2